Kinetic of antioxidant status decay during beef ageing.

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Abstract – Antioxidant mechanisms can preserve meat during processing and storage. Dietary delivery is a strategy to improve antioxidant status in meat, especially if supplements are offered with high quality pastures. Four groups of steers were fed with pasture or grain diets with or without vitamin E supplementation (500 UI/head/day). After slaughter, *Psoas major* samples were packaged under vacuum and submitted to 0, 30, 60 and 90 days of refrigerated storage. Lipid oxidation, as means of TBARS, was higher in grain than in pasture-meat samples ($p<0.05$). Water soluble antioxidants, glutathione and ascorbic acid, were more affected than α-tocopherol and β-carotene during storage. Degradation kinetics of glutathione peroxidase and superoxide dismutase followed a one-order model. Their rates were higher than for catalase, indicating that antioxidants incorporated into the tissue failed to prevent all types of degradation, and that antioxidant enzymes showed different kinetic patterns during meat ageing. Therefore, a combination of dietary strategies and post-harvest technology are necessary in order to improve meat quality and its durability during ageing.

Key Words – Meat, antioxidant enzymes, ageing.

I. INTRODUCTION

Antioxidant mechanisms in meat may preserve its stability during processing and ageing. In vivo, antioxidant enzymes constitute a barrier against oxidative species in muscle, and work together with non-enzyme antioxidants. However, after slaughter, cellular death is triggered, including autophagy, which may release proteases, nucleases, lipases peroxidases producing cell degradation. In this context, fat soluble antioxidants may constitute the principal barrier to stop oxidation, whereas proteins are destroyed (García-Macia *et al.,* [1]).

Concerning incorporation of antioxidants in meat, finishing diet has an important role. Many authors described that vitamin E level is dependent on the dietary source (Gatellier *et al.* [2], Lynch *et al.* [3], and Yang *et al.,* [4], among others).

As far, decay of different antioxidant systems during meat ageing is not widely described. Savary-Auxeloux *et al.* [5] have found that induction of oxidative stress during animal production influenced the total antioxidant capacity but not protein oxidation. In fresh meat, Descalzo *et al.* [6] have described that feeding system influenced non-enzymatic antioxidants but had less effect on the enzymatic system. Renerre *et al.* [7] have shown the decay of Superoxide dismutase (SOD) activity after 8-days refrigerated storage, whereas catalase (CAT) and glutathione peroxidase (GPX) were more stable. In a previous work, we have described that enzymes SOD and CAT did not change significantly during beef storage for nine days under aerobic exposure at 4 °C. Conversely, decrease of GPX activity could be an indicator of beef deterioration (Insani *et al.* [8]). But their activity may decay in different forms after slaughter and post-mortem ageing, as along with tissue disruption. The aim of this work was to describe the progression of oxidative damage during meat ageing for 90 days, in *Psoas major* muscles with different initial contents of vitamin E (issued from different dietary finishing modes) and to test whether higher initial levels of non-enzymatic antioxidants have an impact or not on the overall antioxidant status decay .

II. MATERIALS AND METHODS

A. Animals and diets: Ten crossbreed steers (Hereford x Aberdeen Angus) were grown on pasture (*Medicago sativa* and *Festuca arundinacea S.*) in Pergamino, Buenos Aires province, Argentina, under a rotational system at

an average forage rate of 1500 kg/DM/Ha. Thereafter, five animals were kept on pasture as control (P) and another five (PE) received 500 U/day of vitamin E (all-rac α-tocopherol, Roche Argentina) within concentrate feed (1 kg). Similarly, ten steers were fed with 5 kg corn+ 6 kg hay/day/animal. Whereas five animals remained on grain silage diet (G), the others were supplemented with vitamin E (GE) as stated above. Consume of vitamin E, β-carotene and lutein was estimated from individual components. Steers were slaughtered after reaching 480 kg weight. Muscle samples (*P major*) were harvested and transported to the laboratory, wrapped under vacuum, divided into 4 treatments (each animal) and stored in the dark at 2 °C for 0; 30; 60 and 90 days.
All procedures used complied with national regulations for animal's handling and those of the National Institute for Agricultural Technology (INTA) for the use of experimental animals including welfare.

B. Oxidation determination: Lipid oxidation was determined by Thiobarbituric Acid Reactive Susbtances (TBARS) and expressed as malonaldehyde equivalents per kg muscle. Volatiles were determined by SPME-GC-FID and were calibrated with standard curves of each compound.

C. Fat soluble vitamins: tocopherols and carotenoids were extracted from muscle homogenates with n-hexane, after saponification with KOH-Pyrogallol. Isomers were determined by HPLC-UV/vis and fluorescence detection as described previously by Descalzo *et al.* [6]

D. Antioxidant enzyme assays: ten grams of minced meat were homogenized (Ultraturrax, IKA, Germany at 3000 rpm for 2 min) with phosphate buffer (pH 7.7; 0.05M) and 2% Triton X-100. Two milliliters were extracted with ethanol: chloroform (1:4 vol/vol) for SOD determination. The rest of was centrifuged at 4 ºC at 9500 x g and the supernatant kept at -80 ºC until processing. Total SOD activity was determined according to Misra and Fridovich [9]. CAT activity was measured by the rate of disappearance of 10 mM H_2O_2 at 240 nm as described by Aebi, [10]. GPX activity was assayed with a GSH reduction coupled to a NADPH oxidation by glutathione reductase (Flohé and Günzler, [10]). Enzyme activities were referred to protein content within the homogenate.
Glutathione was determined by measuring formation of TNB from DTNB (5, 5'-dithio-bis 2-nitrobenzoic acid). Total antioxidant activity was determined by FRAP and ascorbic acid was obtained by the same reaction (FRAPC) in the presence of ascorbate oxidase (Descalzo *et al*., [6]). All standards and chemicals were purchased from Sigma-Aldrich Argentina.

E. Statistical analysis: Variables were compared for time and dietary treatment using two-way ANOVA with interaction (XLSTAT Version 2015.1.01).

F. Kinetic modeling: Antioxidant decay was described via a first-order kinetic:
$C=Co\ exp^{-kt}$
With C and Co the concentration in antioxidant at time t and t=0 in day. The rate constant k, in day^{-1}, was identified by non a linear regression method.

RESULTS AND DISCUSSION

Dietary antioxidant intake (DAI) was calculated from daily DM intake and the percentages of individual components in the diet. In average, PE, P, GE and G groups consumed 1813, 1313, 1044 and 544 UI of vit E/head/day, respectively. DAI of tocopherols, correlated positively with the levels in *P major* ($p<0.05$; *R* Pearson = 0.82), whereas lipid oxidation showed an inverse relation (*R* Pearson= -0.747). Moreover, average DAI for β-carotene and lutein were 604 mg and 33.07 g/head/day for both P and PE and 5.65 mg and 1.4 g/head/day for both G and GE groups.
As shown in table 1, the effect of diet had a great impact on antioxidants in fresh meat (day 0). Total antioxidant activity, as well as α-tocopherol, β-carotene and glutathione values were higher in pasture than in grain samples ($p<0.05$). Even ascorbic acid (AA) tended to be higher, in pasture than in grain meat.
However, despite their initial content, they failed to stop degradation, of either enzymatic (table 2) or non-enzymatic antioxidants (table 1) during meat ageing with especially substantial decrease in AA, SOD and GPX for P and PE.

Oxidation parameters resulted overall higher in grain samples than in pasture ($p<0.05$): Supplementation tended to maintain lower quantities of TBARS until day 30 (table 2). Despite their initial values, evolution of TBARS was similar for all treatments (increase and decay). Other indicators of lipid oxidation termination are volatile aldehydes. Their production showed an increment with time ($p<0.05$) but with high variability among diets (table 2). These compounds are very reactive and may be involved in furthest oxidation reactions.

Table 1: Antioxidant parameters in meat during ageing

Diet* time	α-toc (μg/g tej)	β-car (μg/g tej)	GSH (nmoles/g)	FRAP (μM)	AA (μM)
G0	1.20^{cd}	0.07^{d}	324^{b}	175^{abc}	32^{abcd}
G30	0.92^{d}	0.11^{cd}	150^{bcd}	194^{abc}	11^{cd}
G60	0.71^{d}	0.09^{cd}	146^{bcd}	148^{c}	31^{abcd}
G90	0.94^{d}	0.11^{cd}	50^{d}	161^{bc}	21^{bcd}
GE0	2.24^{bc}	0.061^{d}	269^{bc}	170^{abc}	37^{abc}
GE30	1.32^{cd}	0.17^{cd}	169^{bcd}	199^{abc}	8^{d}
GE60	1.23^{cd}	0.07^{cd}	111^{cd}	163^{abc}	21^{bcd}
GE90	1.22^{cd}	0.08^{cd}	75^{d}	153^{c}	20^{bcd}
P0	3.34^{ab}	0.39^{bc}	531^{a}	233^{a}	51^{a}
P30	2.91^{b}	0.58^{ab}	233^{bcd}	172^{abc}	13^{cd}
P60	2.61^{b}	0.53^{ab}	85^{cd}	145^{c}	17^{bcd}
P90	3.08^{ab}	0.51^{ab}	47^{d}	163^{abc}	29^{abcd}
PE0	4.15^{a}	0.52^{ab}	613^{a}	229^{ab}	42^{ab}
PE30	2.85^{b}	0.81^{a}	234^{bcd}	187^{abc}	9^{d}
PE60	2.92^{b}	0.53^{ab}	140^{bcd}	180^{abc}	16^{cd}
PE90	3.13^{ab}	0.62^{ab}	66^{d}	154^{c}	22^{bcd}
p - value	$6\ 10^{-19}$	$3\ 10^{-16}$	$7\ 10^{-18}$	$3\ 10^{-04}$	$5\ 10^{-07}$

Different letters indicate significant differences within the same column (p<005).
α-toc, β-car, GSH, FRAP, AA: α-tocopherol, β-carotene, reduced glutathione, total antioxidant activity and ascorbic acid respectively.

Catalase, SOD, GSH and FRAP showed positive interaction between time and diet ($P<0.05$), whereas the other parameters not. Interestingly, the rate of degradation of water soluble antioxidants (Table 3), as reduced glutathione and ascorbic acid, was higher than the degradation rate of fat soluble antioxidants.

Table 2: Oxidation markers and activity of antioxidant enzymes in meat during ageing

Diet* time	TBARS (mg MDA/kg)	SUM volatiles (ppm)	CAT (U/mg prot)	SOD (U 50% /mg)	GPX
G0	0.27^{bcd}	3.40^{ab}	0.55^{bcd}	18.7^{bc}	53.2^{ab}
G30	0.51^{a}	2.35^{ab}	0.51^{d}	12.8^{cd}	9.9^{d}
G60	0.33^{abc}	3.04^{ab}	0.65^{bcd}	11.3^{cd}	11.6^{d}
G90	0.24^{bcd}	3.76^{ab}	0.53^{bcd}	12.1^{cd}	13.4^{d}
GE0	0.21^{bcd}	2.76^{ab}	0.71^{abcd}	16.4^{cd}	35.9^{bc}
GE30	0.22^{bcd}	1.56^{b}	0.53^{cd}	10.7^{cd}	10.1^{d}
GE60	0.38^{ab}	2.08^{b}	0.55^{bcd}	12.4^{cd}	10.6^{d}
GE90	0.22^{bcd}	2.89^{ab}	0.87^{abcd}	11.3^{cd}	13.9^{d}
P0	0.09^{d}	1.87^{b}	0.61^{bcd}	30.3^{a}	45.9^{ab}
P30	0.29^{bcd}	2.17^{b}	0.50^{d}	15.2^{cd}	11.8^{d}
P60	0.17^{bcd}	2.20^{b}	1.06^{abc}	9.2^{d}	20.8^{cd}
P90	0.09^{d}	1.87^{b}	1.07^{ab}	10.0^{d}	14.8^{d}
PE0	0.08^{d}	2.57^{ab}	0.71^{abcd}	25.6^{ab}	58.5^{a}
PE30	0.15^{cd}	1.78^{b}	0.44^{d}	14.2^{cd}	13.5^{d}
PE60	0.15^{cd}	3.04^{ab}	0.72^{abcd}	10.4^{d}	14.9^{d}
PE90	0.08^{d}	4.63^{a}	1.21^{a}	9.7^{d}	16.8^{cd}
p - value	$2\ 10^{-09}$	$9\ 10^{-06}$	$5\ 10^{-6}$	$1\ 10^{-14}$	$7\ 10^{-16}$

Different letters indicate significant differences within the same column (P<005).
TBARS, SUM, CAT, SOD, GPX: Thiobarbituric acid reactive substances, Sum of volatiles (3-methyl-butanal, pentanal, hexanal, heptanal and octanal as main species), catalase, superoxide dismutase and glutathione peroxidase respectively.

β-carotene levels remained quite constant for all treatments during ageing under the assayed conditions (rate constants below $1\ 10^{-7}$day^{-1}), whereas α-tocopherol was consumed more rapidly (orders 10^{-3}). α-tocopherol degradation rates were higher in supplemented diets (GE and PE).
Probably, α-tocopherol constitutes, together with AA and GSH that may regenerate it, the first barrier against free radicals so it is consumed more effectively than β-carotene.
Antioxidant enzymes, SOD and GPX, also decreased (table 3) and reached their basal activity between 30 and 60 days of storage. GPX descended at a similar rate as did the co-factor GSH, as well as augmented the formation of

GSSG (oxidized form, data not shown). Therefore, loss of activity may be attributed to starving of co-factors as well as protein damage. On the contrary, CAT activity was less affected by degradation processes.

Table 3: Degradation rate constant k (day^{-1}) of non-enzymatic antioxidants and antioxidant enzymes.

Parameter	G	GE	P	PE
α-toc	$5.09\ 10^{-03}$	$9.37\ 10^{-03}$	$2.08\ 10^{-03}$	$4.90\ 10^{-03}$
β-car	$3.19\ 10^{-13}$	$2.17\ 10^{-08}$	$5.45\ 10^{-11}$	$2.59\ 10^{-12}$
AA	$5.24\ 10^{-03}$	$1.27\ 10^{-02}$	$1.72\ 10^{-02}$	$1.80\ 10^{-02}$
FRAP	$1.12\ 10^{-03}$	$5.46\ 10^{-04}$	$5.92\ 10^{-03}$	$4.52\ 10^{-03}$
GSH	$1.86\ 10^{-02}$	$1.47\ 10^{-02}$	$2.84\ 10^{-02}$	$2.78\ 10^{-02}$
CAT	$1.50\ 10^{-12}$	$2.32\ 10^{-04}$	$1.29\ 10^{-13}$	$2.54\ 10^{-14}$
SOD	$6.92\ 10^{-03}$	$5.35\ 10^{-03}$	$1.77\ 10^{-02}$	$1.41\ 10^{-02}$
GPX	$3.71\ 10^{-02}$	$2.24\ 10^{-02}$	$1.95\ 10^{-02}$	$2.96\ 10^{-02}$

These results indicate that natural antioxidants incorporated into the tissues cannot avoid the degradation of enzymatic and non-enzymatic antioxidants, but may preserve meat from oxidation by maintaining lower levels of oxidative species. However, this conclusion must be balanced considering the ageing time. Indeed, the fact that volatile compounds augmented significantly in PE samples along storage, deserves further considerations due to the possibility of fat soluble antioxidants to became pro-oxidant themselves when recycling mechanisms for free radicals are disrupted due to degradation processes within tissues.

III. CONCLUSION

Dietary delivery of antioxidants is a strategy to increase overall oxidative stability of meat. However, stability is not equal for all antioxidants. Water soluble factors, as reduced glutathione and ascorbic acid, decayed more rapidly than α-tocopherol and β-carotene during meat ageing under refrigerated vacuum. Glutathione peroxidase and superoxide dismutase were more affected than catalase, indicating differences at the enzymatic level. These findings are important in terms of combination of dietary strategies and post-harvest technology in order to improve meat quality and its durability during ageing.

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